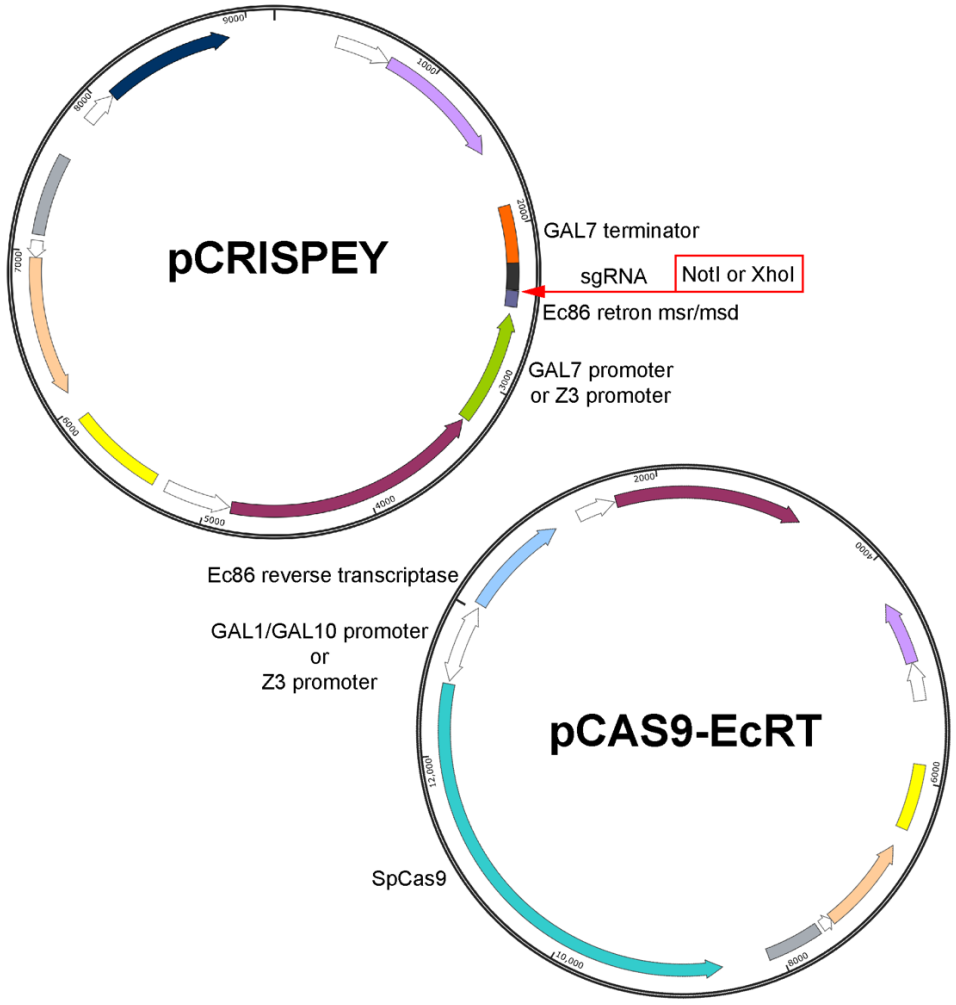
THE EXPANDED CRISPEY KIT

This kit contains a suite of plasmids for creating genomic edits in *Saccharomyces cerevisiae* using CRISPR/Cas9 cleavage coupled with directed homologous recombination to repair Cas9 breaks. A variety of mutation types can be introduced into the genome using this system, including deletions and point mutations. Introducing genomic insertions should also be possible, but it has not been tested by the authors. This kit can be used in laboratory, wild, and industrial strains that are haploid or diploid.



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References

Reference for expanded CRISPEY kit (plasmids described in this manual):

Tara N. Stuecker, Stephanie E. Hood, Julio Molina Pineda, Sonali Lenaduwe, Joshua Winter, Meru J. Sadhu, Jeffrey A. Lewis. **Improved vectors for retron-mediated CRISPR-Cas9 genome editing in *Saccharomyces cerevisiae***. G3 Genes|Genomes|Genetics. 2025. Volume 15. Issue 10.

All plasmids are derived from the original CRISPEY system described here:

Eilon Sharon, Shi-An A. Chen, Neil M. Khosla, Justin D. Smith, Jonathan K. Pritchard, Hunter B. Fraser. **Functional Genetic Variants Revealed by Massively Parallel Precise Genome Editing**. Cell. 2018. Volume 175. Issue 2. Pages 544-557.e16.

Reference for CRISpy-Pop gRNA design tool:

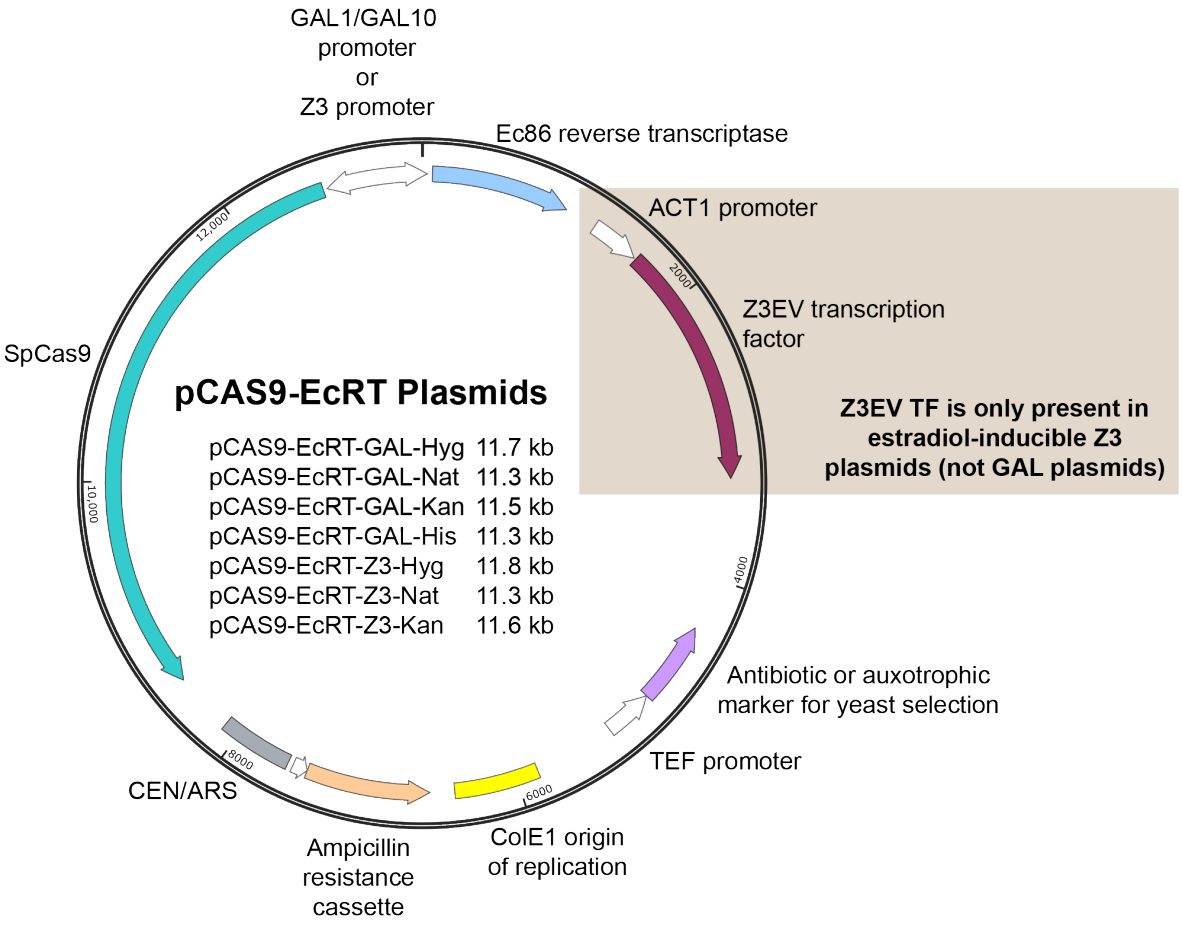
Hayley R Stoneman, Russell L Wrobel, Michael Place, Michael Graham, David J Krause, Matteo De Chiara, Gianni Liti, Joseph Schacherer, Robert Landick, Audrey P Gasch, Trey K Sato, Chris Todd Hittinger. **CRISpy-Pop: A Web Tool for Designing CRISPR/Cas9-Driven Genetic Modifications in Diverse Populations**. G3. 2020. Volume 10. Issue 11. Pages 4287–4294.

How the System Works

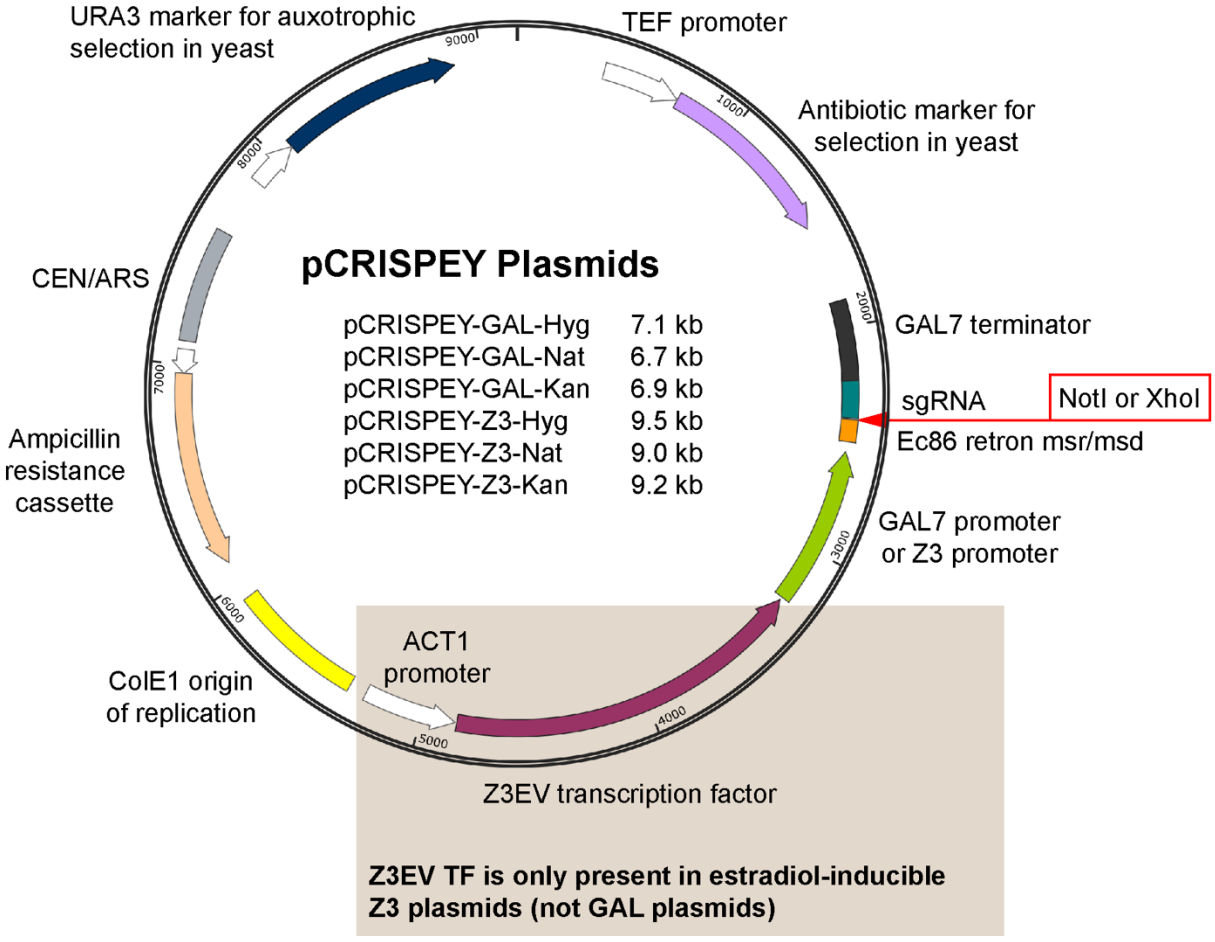
This system uses a method called Cas9 Retron precISe Parallel Editing via homologY (CRISPEY) developed by *Sharon et. al* (citation on page 1), which employs CRISPR/Cas9 to precisely cut the genome at a desired location coupled with a bacterial retron to amplify a specific repair template *in vivo*. This approach greatly increases the frequency of homologous recombination to repair the break induced by Cas9 cleavage, allowing the researcher to create site-specific edits in the yeast genome.

The Expanded CRISPEY system is composed of two plasmid types, a pCas9-EcRT plasmid paired with a pCRISPEY plasmid.

The pCas9-EcRT plasmids express: 1) *S. pyogenes* Cas9 under control of either the GAL1 promoter or the bi-directional Z_3 promoter, 2) yeast codon-optimized *E. coli* Ec86 retron reverse transcriptase under control of either the GAL10 promoter or the bi-directional Z_3 promoter, 3) an ampicillin resistance cassette for selection in *E. coli*, and 4) an MX antibiotic or auxotrophic cassette for selection in yeast. The MX cassette consists of a yeast antibiotic resistance gene or auxotrophic marker flanked by the TEF promoter and TEF terminator. Estradiol-inducible (Z3) plasmids also express the Z3EV transcription factor under the control of the ACT1 promoter.



The pCRISPEY plasmids have a unique restriction site for cloning desired gRNA and repair template sequences. pCRISPEY-GAL plasmids use NotI, while pCRISPEY-Z3 plasmids use XhoI. Your gRNA sequence will be fused to the structural guide RNA (sgRNA) in the plasmid to direct CRISPR/Cas9 cleavage to your desired location in the genome. Your repair template will be fused to a the Ec86 Retron msr and 5'msd, allowing the repair template to be amplified *in vivo* by the Ec86 reverse transcriptase. This amplification of the repair template increases the frequency of homologous recombination to create your desired edit. Expression of both the gRNA and repair template are driven by either the GAL7 promoter (galactose-inducible plasmids) or the Z3 promoter (estradiol-inducible plasmids). Each plasmid contains two options for selection in yeast: 1) URA3 for auxotrophic selection, or 2) an MX antibiotic cassette for antibiotic selection. Estradiol-inducible (Z3) plasmids also express the Z3EV transcription factor under the control of the ACT1 promoter.



Plasmids Included in Kit

Cas9 Plasmids

Plasmid Name	Markers for Yeast Selection	Induced by	Addgene ID#
pCas9-EcRT-GAL-Nat	NatMX	Galactose	232090
pCas9-EcRT-GAL-Hyg	HygMX	Galactose	232091
pCas9-EcRT-GAL-Kan	KanMX	Galactose	232092
pCas9-EcRT-GAL-HIS3	HIS3MX	Galactose	232093
pCas9-EcRT-Z3-Nat	NatMX	β-estradiol	232094
pCas9-EcRT- Z3-Hyg	HygMX	β-estradiol	232095
pCas9-EcRT- Z3-Kan	KanMX	β-estradiol	232096

CRISPEY plasmids for cloning gRNA and repair template

Plasmid Name	Markers for Yeast Selection	Induced by	Addgene ID#
pCRISPEY-GAL-Nat	NatMX or URA3	Galactose	232097
pCRISPEY-GAL-Hyg	HygMX or URA3	Galactose	232098
pCRISPEY-GAL-Kan	KanMX or URA3	Galactose	232099
pCRISPEY-Z3-Nat	NatMX or URA3	β-estradiol	232100
pCRISPEY-Z3-Hyg	HygMX or URA3	β-estradiol	232101
pCRISPEY-Z3-Kan	KanMX or URA3	β-estradiol	232102

Control CRISPEY plasmids expressing gRNA and repair template for frameshift in ADE2

Plasmid Name	Markers for Yeast Selection	Induced by	Addgene ID#
pCRISPEY-GAL-Nat-ADE2	NatMX or URA3	Galactose	232103
pCRISPEY-GAL-Hyg-ADE2	HygMX or URA3	Galactose	232104
pCRISPEY-GAL-Kan-ADE2	KanMX or URA3	Galactose	232105
pCRISPEY-Z3-Nat-ADE2	NatMX or URA3	β-estradiol	232106
pCRISPEY-Z3-Hyg-ADE2	HygMX or URA3	β-estradiol	232107

All plasmids contain an ampicillin resistance gene for selection in *E. coli*

Primers Needed

Primers are not included in kit. They can be purchased from any DNA synthesis company (IDT, Genscript, etc). Primers can be purchased at the lowest scale with standard desalting.

Primers for amplifying gRNA and repair template oligo to create insert for cloning into pCRISPEY vectors. These primers are only needed if using option 1 to clone gRNA and repair template. If using the direct cloning method (option 2), these primers are not necessary.

Primer Name	**Sequence (5' → 3')**
CRISPEY_F	CGGCATCCTGCATTGAATCTGAGTTACTGTCTGTTTTCCTGGGTCACGCGTAGGA
CRISPEY_R	ATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGAAAC

Primers for screening pCRISPEY clones to verify presence of gRNA/repair template insert after Gibson cloning step.

Primer Name	**Sequence (5' → 3')**
pCR_GAL_Ins_Chk_F	TTTTTGTGATGCTCGTCAGG
pCR_Z3_Ins_Chk_F	GTTTGTATTACTTCTTATTCAAATGTCA
pCR_Ins_Chk_R	CGCACCCTTACGTCAGAAGA

Primers for sequencing gRNA/repair template inserts in pCRISPEY vectors. All sequencing primer options are listed below, but any primer can be used to sequence any pCRISPEY vector. See map in "Screening pCRISPEY Clones for Inserts" section below for annealing locations and sequencing directions of each primer.

Primer Name	**Sequence (5' → 3')**
CRISPEY_Retron_SEQ_F	GCATCTGATGAGTCCGTGAG
CRISPEY_SEQ_R	GGACCATGCCGGCCATCAAAA
T7 promoter	TAATACGACTCACTATAGGG
M13 forward	TGTAAAACGACGGCCAGT

Designing Your Guide RNA and Repair Template for Cloning into pCRISPEY

Option 1 – Cloning amplified gRNA/repair template

Your guide RNA (gRNA) and repair template will be synthesized as a single oligo (IDT ultramer or similar) or as a DNA fragment (IDT gBlock or similar). See below for guidelines on designing gRNA and repair template sequences. Your final ultramer/gBlock sequence will look like this…

5’ Primer annealing region Retron 3’ msd sequence
5’-GGGTCACGCGTAGGA [repair template] AGGAAACCCGTTTCTTCTGACGTAAGGGTGCGCA

3’ Primer annealing region
[gRNA] GTTTCAGAGCTATGCTGGAAAC-3’

This synthetic DNA fragment will be used as template for PCR amplification. The CRISPEY_F and CRISPEY_R primers amplify your gRNA and repair template oligo/gBlock, adding 40 nt of vector sequence to each end. These vector sequences correspond to the 40 nt upstream and 40 nt downstream of the NotI or XhoI cloning site in the pCRISPEY vectors. The addition of these vector sequences by PCR allows the gRNA and repair template oligo/gBlock to be fused with the linearized pCRISPEY vector using Gibson cloning.

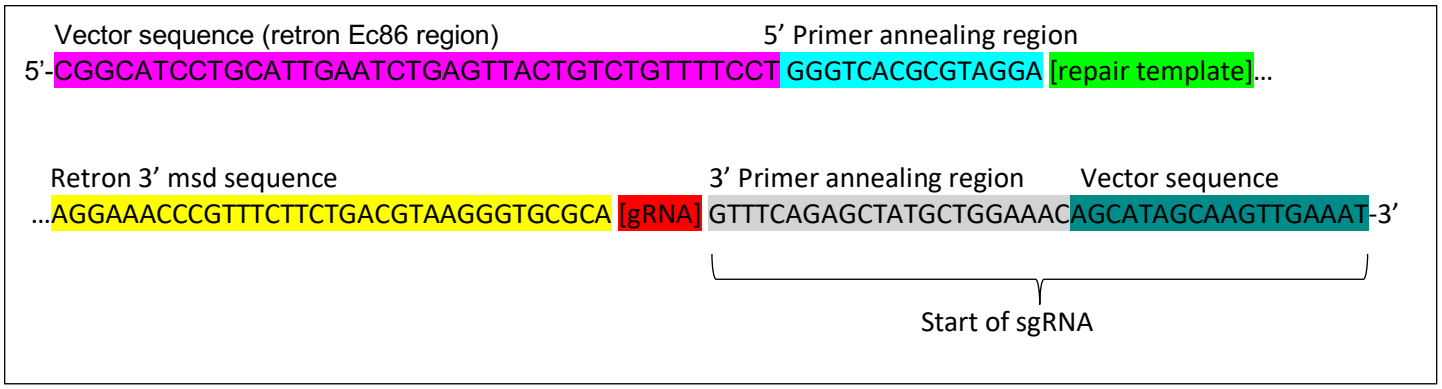
CRISPEY-F
Vector sequence (retron Ec86 region) 5’ Primer annealing region
5’-CGGCATCCTGCATTGAATCTGAGTTACTGTCTGTTTTCCTGGGTCACGCGTAGGA-3’

The CRISPEY-F primer has 40 nt of vector sequence for Gibson cloning followed by the 15 nt 5’ primer annealing region, which is identical to the start of your ultramer/gBlock. The 5’ primer annealing region allows the primer to anneal the ultramer/gBlock, but this sequence is arbitrary. It can be changed if desired, however you *must* change the sequence in *both* the forward primer and the ultramer/gBlock if you wish to alter this sequence.

CRISPEY-R
Vector sequence (sgRNA) 3’ Primer annealing region (part of sgRNA)
5’-ATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGAAAC-3’

The CRISPEY-R primer has 40 nt of vector sequence for Gibson cloning, with the last 22 nt matching the 3’ primer annealing region of the ultramer/gBlock. **This primer annealing region cannot be changed**. This primer creates a fusion of the gRNA target sequence you design with the structural gRNA sequence required for CRISPR/Cas9 cutting.

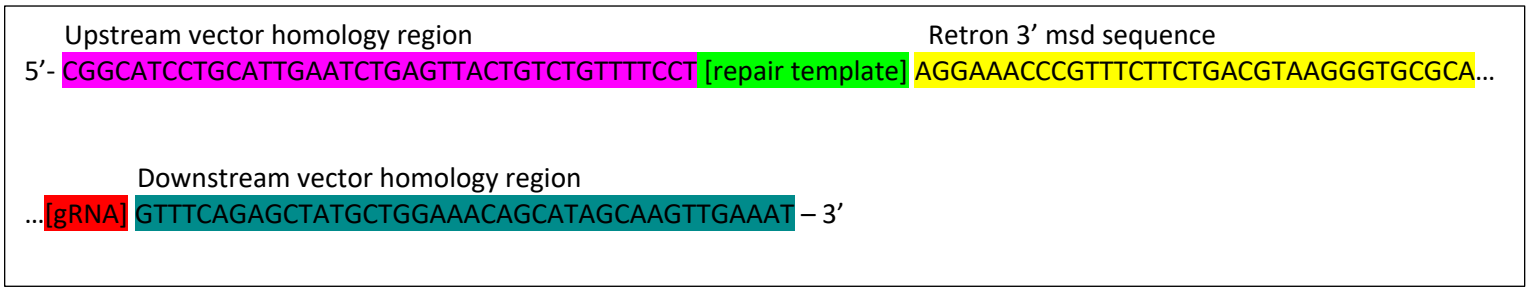
Your final PCR product, which will be used as insert for Gibson cloning, will look like this…



Option 2 – Direct cloning of gRNA/repair template

Alternatively, a single double stranded DNA fragment (IDT gBlock or similar) can be ordered containing the gRNA, repair template and flanking vector sequences needed for Gibson cloning. A single stranded DNA oligo will not work for this method. For direct cloning, the amplification step that adds the vector sequences for Gibson cloning is not necessary because the Gibson homology ends are included in your gBlock sequence. The DNA fragment can be directly cloned into the XhoI or NotI linearized pCRISPEY vector. See below for guidelines on designing gRNA and repair template sequences.

For this method, your gBlock would look like this…



Designing your gRNA sequence

The gRNA sequence will direct Cas9 to cut the genomic DNA at your desired location. We recommend using the online tool CRISpy-Pop (https://crispy-pop.glbrc.org/) to design your gRNA with the following settings:

- *Strain*: Select the strain you plan to mutate. If you plan to mutate more than one strain, you can just select any one of them for this step.
- *Gene*: Select the gene you plan to mutate.
- *PAM Sequence*: NGG
- *Target Type*: Gene
- *Spacer Length*: 20

The CRISpy-pop output will give you a list of possible gRNAs in order of activity score. Higher predicted activity scores tend to have higher cutting efficiencies. Other factors to consider when choosing your gRNA from the list:

- Position in gene: this is the position in the target gene where Cas9 will cleave the DNA
    - If you are creating a gene knockout by inserting a frameshift or stop codon, you want to select a position towards the beginning of the gene.
    - If you are creating a gene knockout by removing an entire ORF or a large portion of the gene, select a position in the middle of the portion being removed.
    - If you are introducing a point mutation, select the gRNA with the cut site closest to the nucleotide you are trying to change.
- Strains covered: this is how many strains in their database would be cut by gRNA
    - If you will be using your gRNA for multiple strains, you can click on the individual gRNAs to see a list of strains that would be cut by that gRNA. Check to make sure all the strains you need are on the list. Note that not all yeast strains are in the CRISpy-pop database.
- Spacer length: this is the length of the gRNA
    - Different lengths of gRNAs have been shown to work in yeast. We have had great success using 20 bases, but this parameter can be adjusted if desired.
- Strand: this indicates if the gRNA is located on the coding (+) or non-coding (-) strand.
    - In general, it is easier to use a gRNA located on the coding strand whenever possible.
- PAM: protospacer adjacent motif; this is the NGG immediately following the gRNA
    - Cas9 will cut 3 nt upstream of the PAM
    - Note the PAM sequence of the gRNA you select. You will need it for the repair template design.

IMPORTANT

Do NOT include the PAM in your oligo. Only include the 20 bp gRNA.

Designing Your Repair Template Sequence

Your repair template will be 100 nt long, including 50 nt of homology on either side of your introduced mutation. It is theoretically possible to have a longer repair template, however the authors have not tested larger templates at the time of publication.

You must introduce a mutation to disrupt the PAM sequence in your repair template. Otherwise, Cas9 will continue to cut the genome, even after the gene has been repaired. If this occurs, you will end up with an undesired mutation because Cas9 will continue cutting the genome until a mutation occurs by chance that disrupts the PAM. It will also allow Cas9 to cut your pCRISPEY plasmid, reducing the efficiency of editing. The PAM sequence is the NGG immediately following the gRNA. It will be listed in the CRISpy-pop gRNA list. Take special care in identifying the PAM if your gRNA is located on the non-coding strand. Make sure you are reading the correct strand for your specific gRNA.

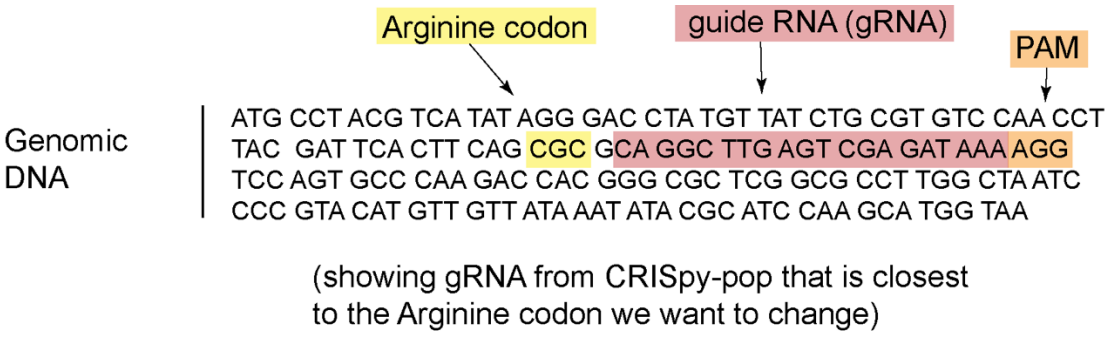
If you are creating a gene disruption by introducing a frameshift mutation or stop codon, select the 50 nt upstream of your mutation site and the 50 nt downstream of your mutation site. Make sure to include your desired mutation in the repair template sequence. Don't forget to mutate the PAM sequence, which can be done as part of your frameshift mutation or as a separate edit.

If you are creating a gene disruption by deleting the entire gene or a large portion of the gene, your repair template will be 50 nt upstream and 50 nt downstream of the region you wish to remove. In this case, you don't have to worry about the PAM sequence because it will be deleted during repair.

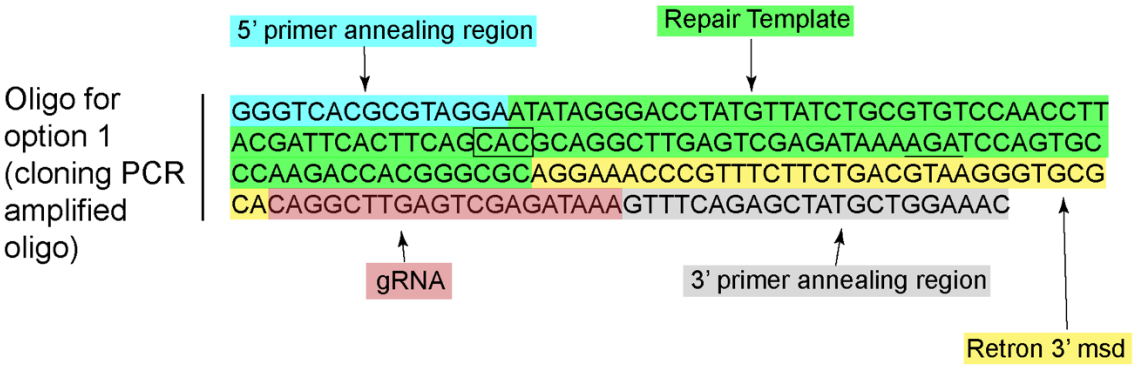
If you are creating a point mutation, your repair template will be the sequences 50 nt upstream and 50 nt downstream of the mutation being introduced. Make sure to include your desired mutation in the repair template sequence. When altering a codon in an ORF, select a codon for your mutant that is closest to the same codon usage as the codon being replaced. If you replace a common codon with a rare codon, for example, it may affect translation efficiency of your protein. Try to match the codon usage of your altered codon to the original codon as closely as possible. Don't forget to also mutate the PAM site. If your introduced mutation disrupts the PAM, no further action is required. If your mutation does not affect the PAM site, a separate mutation must be included in your repair template to disrupt the PAM. In this case, you want to make sure the PAM mutation is silent and will not change the coding sequence of the protein. It is highly recommended to create a second control strain where you introduce the silent PAM mutation but do not disrupt any other sequence in your gene. This is done by creating a second template oligo that contains the same gRNA as your point mutant oligo, but only has the PAM disruption in the repair template (not your point mutation). After creating this mutation in yeast, the strain can be used as a negative control to prove that the silent mutation disrupting the PAM has no effect on your phenotype of interest.

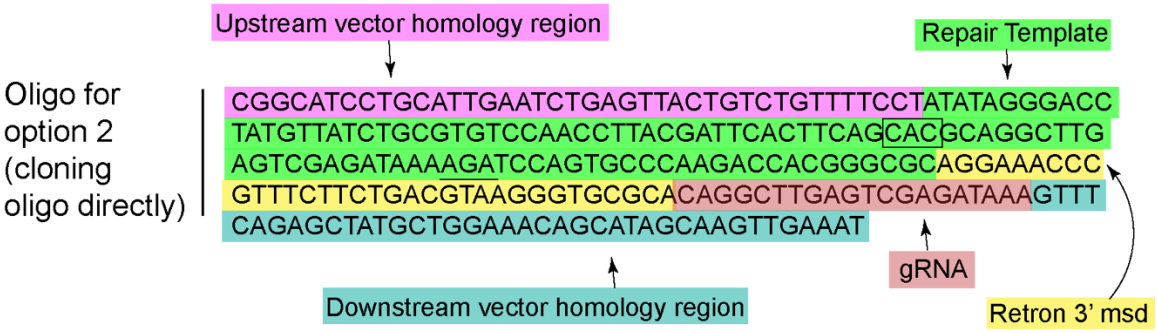
Example Design for a Point Mutation

For this example, we will change a CGC (Arginine) codon to CAC (Histidine).



(showing gRNA from CRISpy-pop that is closest to the Arginine codon we want to change)





IMPORTANT NOTES

- Guide RNA in oligo does NOT include PAM site.
- PAM site (AGG) is mutated to AGA in the repair template (silent mutation- both code for the same codon). This is underlined in above example.
- Desired edit (Arginine to Histidine muation) is boxed in example above.

Adding Barcodes (optional)

If you wish to add barcodes or other unique identifying features to your plasmids, they can be added to your insert, but the exact method will vary depending on the cloning method you will use to insert your gRNA and repair template.

Barcodes can either be specific (each plasmid has the same user-defined sequence) or random (each plasmid has a unique sequence) or a combination of the two strategies. To insert a random barcode, designate the nucleotides of the barcode as N's.

For the amplification method (option 1), barcodes or unique sequences can be added to the CRISPEY-F primer between the vector sequence and 5'-annealing portions of the primer. The ultramer/gBlock does not require alteration.

For the direct method (option 2), barcodes can be added directly to the ultramer/gBlock between the upstream vector homology region and the repair template.

Note Regarding Antibiotic Combinations

There is a slight decrease in editing efficiency when the Kan and Hyg markers are used together compared to other antibiotic combinations (Kan+Nat or Hyg+Nat). This decrease is observed regardless of which vector carries the Kan vs Hyg marker and was observed for both the galactose-inducible and estradiol-inducible systems. If plasmids encoding the Kan and Hyg markers are used together, it is recommended to increase the induction time to 72 hr to increase editing efficiency.

Cloning gRNA and Repair Template into CRISPEY Vector

Option 1 – Cloning amplified gRNA/repair template

1. Resuspend your synthetic gRNA/repair template (ultramer, gBlock or similar) according to manufacturer instructions. Then, prepare a working dilution at 50 ng/ul.

2. Amplify gRNA/repair template with CRISPEY_F and CRISPEY_R primers to add Gibson cloning ends onto your gRNA/repair template construct. If you have designed your own primers to add barcodes, use your primers instead. The following conditions are specific for the Takara CloneAmp HiFi PCR Premix (Takara cat# 639298), but amplification can be done with any high-fidelity polymerase.

   PCR Components

2x CloneAmp HiFi PCR Premix	20.0 ul
10 uM CRISPEY_F primer	1.2
10 uM CRISPEY_R primer	1.2
50 ng/ul gRNA/repair template DNA	2.0
Nuclease-free water	15.6
	40.0 ul total

   Cycling Conditions

   98°C for 1 min
   35 cycles of:
      98°C for 10 sec
      55°C for 15 sec
      72°C for 1 min
   72°C for 5 min

3. Run 5 ul of PCR product on a gel to ensure that you get a single band of the correct size (249 bp unless a repair template other than 100 bp was used).

4. Purify PCR product with any column-based PCR cleanup kit. Gel extraction is not necessary if you have a single band. Elute in the minimal volume possible for your kit using nuclease-free water.

5. If using pCRISPEY-GAL plasmid, digest with NotI to linearize vector. If using pCRISPEY-Z3 plasmid, digest with XhoI. The following conditions are specific for ThermoFisher FastDigest enzymes, but any brand of restriction enzyme can be used.

   10 ul 10x FastDigest Buffer
   X 5 ug pCRISPEY DNA
   5 NotI or XhoI Enzyme (1 U/ul)
   X Nuclease-free water (to 100 ul total volume)

   Incubate reaction overnight (18-22 hrs) at 37°C, preferably in a thermal cycler with heated lid to prevent sample evaporation.

6. Purify linearized vector with any column-based PCR cleanup kit. Elute in the minimal volume possible using nuclease-free water.

7. Fuse purified gRNA/repair template PCR product into linearized pCRISPEY vector using Gibson cloning. The following conditions are for the NEBuilder® HiFi DNA Assembly kit (cat# E5520S), which is highly recommended. However, other Gibson cloning enzymes can be used if desired. This protocol uses quarter reactions to conserve money.

   Cloning Reaction
   2.5 ul NEBuilder® HiFi DNA Assembly Master Mix
   X ul 25 ng linearized pCRISPEY vector
   X ul 5 ng gRNA/repair template insert DNA
   X ul Nuclease free water (to 5 ul total volume)

   Vector-only Control Reaction (VOC)
   2.5 ul NEBuilder® HiFi DNA Assembly Master Mix
   X ul 25 ng linearized pCRISPEY vector
   X ul Nuclease free water (to 5 ul total volume)

   Incubate reactions for 1 hr at 50°C, preferably in a thermal cycler with heated lid to prevent sample evaporation.

8. Transform 2 ul of each reaction into 50 ul of chemically competent *E. coli* cells (commercial or homemade) using protocol specific to your competent cells.

9. Plate transformed cells onto LB + Ampicillin (100 ug/ml) plates. We recommend plating 100 ul of undiluted cells and 100 ul of a 1:20 dilution.

Option 2 – Direct cloning of gRNA/repair template protocol

1. Resuspend your synthetic gRNA/repair template (gBlock or other) according to manufacturer instructions. Create a working dilution of 5 ng/ul.

2. If using pCRISPEY-GAL plasmid, digest with NotI to linearize vector. If using pCRISPEY-Z3 plasmid, digest with XhoI. The following conditions are specific for ThermoFisher FastDigest enzymes, but any brand of restriction enzyme can be used.

10 ul	10x FastDigest Buffer
X	5 ug pCRISPEY DNA
5	NotI or XhoI Enzyme (1 U/ul)
X	Nuclease-free water (to 100 ul total volume)

   Incubate reaction overnight (18-22 hrs) at 37°C, preferably in a thermal cycler with heated lid to prevent sample evaporation.

3. Fuse gRNA/repair template gBlock into digested pCRISPEY vector using Gibson cloning. The following conditions are for the NEBuilder® HiFi DNA Assembly kit (cat# E5520S), which is highly recommended. However, other Gibson cloning enzymes can be used if desired.

   Cloning Reaction

2.5 ul	NEBuilder® HiFi DNA Assembly Master Mix
X ul	25 ng linearized pCRISPEY vector
1 ul	5 ng gRNA/repair template DNA (gBlock; 5 ng/ul stock)
X ul	Nuclease free water (to 5 ul total volume)

   Vector-only Control Reaction (VOC)

2.5 ul	NEBuilder® HiFi DNA Assembly Master Mix
X ul	25 ng linearized pCRISPEY vector
X ul	Nuclease free water (to 5 ul total volume)

   Incubate reactions for 1 hr at 50°C, preferably in a thermal cycler with heated lid to prevent sample evaporation.

4. Transform 2 ul of each reaction into 50 ul of chemically competent *E. coli* cells (commercial or homemade) using protocol specific to your competent cells.

5. Plate transformed cells onto LB + Ampicillin (100 ug/ml) plates. We recommend plating 100 ul of undiluted cells and 100 ul of a 1:20 dilution.

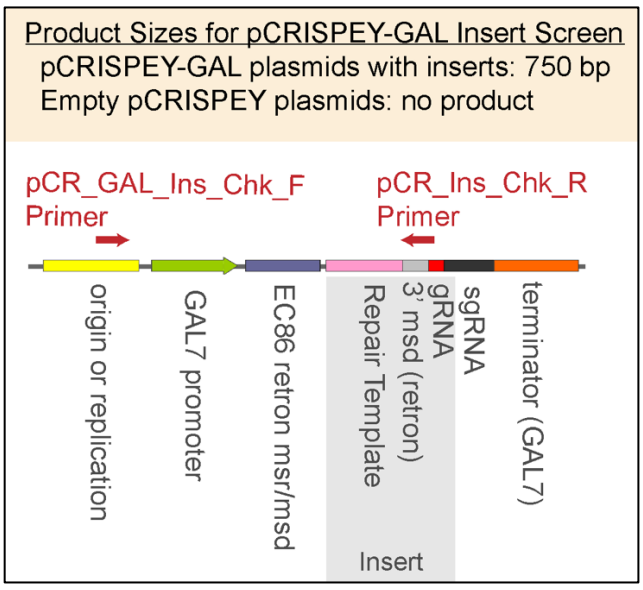
Verification of pCRISPEY Clones

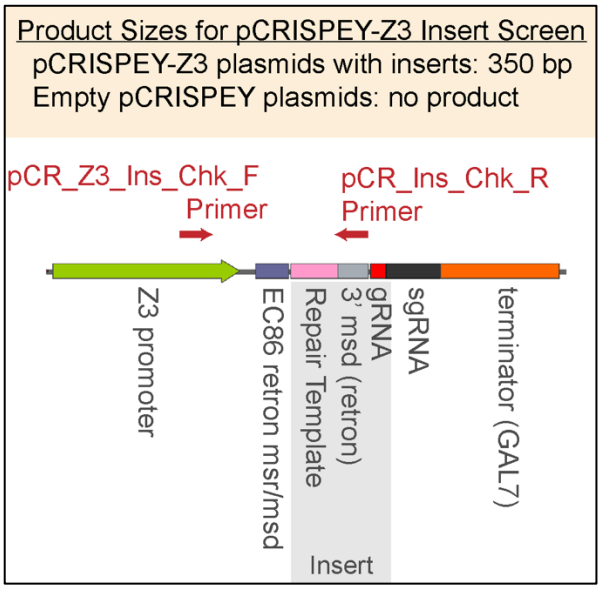
Depending on your cloning efficiency, we recommend screening 5-10 clones for inserts by PCR followed by sequencing the inserts or whole plasmids for 1-2 clones with verified inserts.

Step 1 – Screen clones for inserts

You can get an idea of your cloning efficiency by comparing the number of colonies on your cloning plate to the number of colonies on your vector-only control (VOC) plate. If you have ≥ 5 fold more colonies on your cloning plate compared to the VOC plate, your cloning likely has a high efficiency and you should only need to screen 4-5 clones to find several with inserts. If you still have more colonies on your cloning plate than your VOC plate, but there is less than a 5 fold difference, you may want to screen a higher number of colonies by PCR (~8-10).

The primers for screening plasmid inserts are shown below. The forward primers are unique for the pCRISPEY-GAL or pCRISPEY-Z3 vectors and each anneals upstream of the insertion site. There is a single reverse primer that works with both pCRISPEY-GAL and pCRISPEY-Z3 vectors. The reverse primer binds inside the Ec86 retron 3' msd region. This sequence is part of the invariant section of the oligo insert, located between the repair template and gRNA. Since this sequence is only present if an insert is cloned into the pCRISPEY vector, you should only get a product if an insert was cloned.





1. If colonies are small, they can be patched to a new LB + ampicillin plate and grown overnight at 37°C to get more biomass for screening.

2. It is highly recommended to include both a positive and negative control sample in your PCR when screening for inserts. Make sure your control plasmids use the same induction system as the clones you are screening because the Z3 and GAL systems use different forward primers for the insert screen PCR.
    - Positive control – any control pCRISPEY vector containing the ADE2 gRNA/repair template insert. Alternatively, any pCRISPEY vector containing any gRNA/repair template insert can be used as a positive control.
    - Negative control – the empty pCRISPEY vector you used for cloning your insert. Alternatively, a colony from the VOC plate can be used as a negative control.

3. Templates for PCR can either be prepared by boiling cells in TE + 0.1% Triton X-100 or by purifying plasmids with any standard plasmid prep kit. If using purified plasmids, prepare a 20 ng/ul dilution of each plasmid template. If doing colony PCR, here is a brief protocol for preparing lysed cell PCR templates:
    - For each colony you wish to screen, prepare a 1.7 ml microcentrifuge tube with 100 ul of TE + 0.1% Triton X-100. A stock of TE + 0.1% (v/v) Triton X-100 can be made and stored at room temperature for up to 1 year.
    - Using a sterile pipette tip, scrape a portion of the first colony you wish to screen off the plate and resuspend it in one of the microcentrifuge tubes containing TE + Triton X. The amount of biomass transferred should be somewhere between the size of a pin head and half the size of a pea.
    - Repeat for all samples you wish to screen, resuspending only one colony per tube.
    - Vortex samples vigorously to ensure cells are fully resuspended.
    - Boil tubes in a heat block at >90°C for 10 min to lyse cells.
    - Centrifuge tubes at maximum speed in microcentrifuge to pellet cell debris.
    - Place tubes on ice. If using samples for PCR right away (within 1 hr), you can use them directly. If samples will be stored on ice for more than 1 hr, transfer supernatants to fresh tubes. The pelleted cell debris can re-dissolve over time, which can interfere with your PCR. Do not store samples more than 1 day. It is best to prepare templates just before doing PCR.

4. Prepare a PCR master mix using the following components. To make a master mix, multiply the volumes in the table below by the number of samples being screened plus the number of control samples plus 1 extra to account for pipetting error.

Component	Volume per reaction (ul)
5x Taq Buffer	4
dNTP Mix (2 mM each)	2
Forward Chk Primer (10 uM)	1
pCR_Ins_Chk_R Primer (10 uM)	1
Taq Polymerase	0.5
Nuclease-free water	10.5

pCR_GAL_Ins_Chk_F
for pCRISPEY-GAL plasmids

pCR_Z3_Ins_Chk_F
for pCRISPEY-Z3 plasmids

5. Mix master mix thoroughly by pipetting up and down or pulse vortexing. Aliquot 19 ul of master mix into PCR tubes.

6. Add 1 ul of template DNA to each tube (either lysed cells or 20 ng/ul dilutions of purified plasmid DNA).

7. Run the following PCR program:

94°C for 4 min
35 cycles of
    94°C for 30 sec
    55°C for 45 sec
    72°C for 1 min
72°C for 5 min

8. Run 5 ul of each PCR product on an agarose gel to determine which clones have inserts.

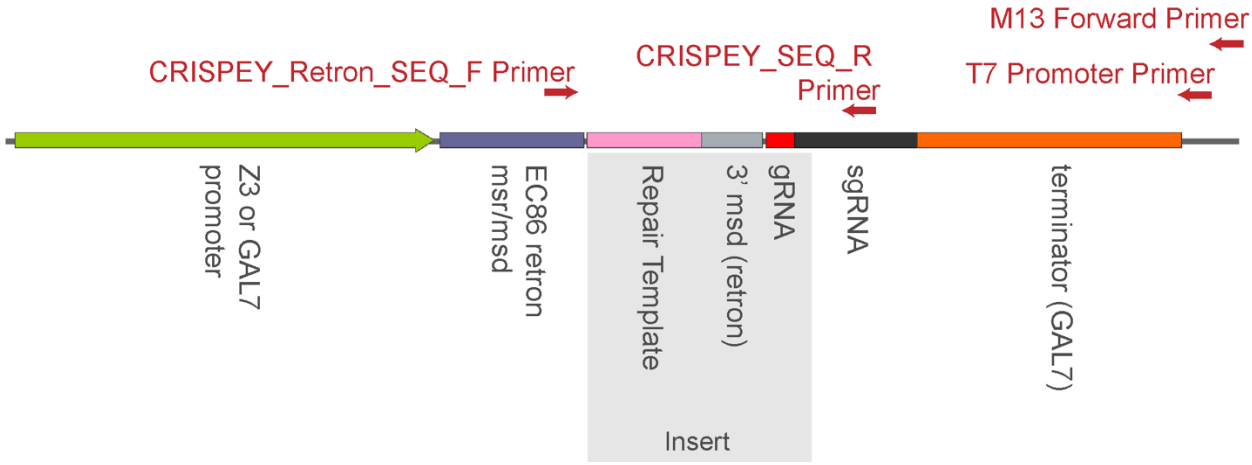
For pCR_**GAL_**Ins_Chk_F + pCR_Ins_Chk_R primer pair:
- Expect 750 bp product if gRNA/repair template oligo is inserted into pCRISPEY-GAL vector.
- Expect no band if vector is empty (no oligo insert is present).

For pCR_**Z3**_Ins_Chk_F + pCR_Ins_Chk_R primer pair:
- Expect 350 bp product if gRNA/repair template oligo is inserted into pCRISPEY-Z3 vector.
- Expect no band if vector is empty (no oligo insert is present).

Step 2 – Sequence inserts

Following insert verification, we recommend sequencing the insert of 1-2 clones to find a clone free of unwanted mutations. There are several options for sequencing primers, which are shown below with locations and directions indicated. Since the inserts are small, it is only necessary to sequence in one direction. An alternative option is Nanopore sequencing of the entire plasmid (Plasmidsaurus or similar service), in which case no primer is necessary.

Select a clone that has the correct sequence for the promoter region, Ec86 retron sequences, repair template, gRNA, and sgRNA.



Introducing Mutation into Yeast by CRISPEY/Cas9-Mediated Cutting and Directed Homologous Recombination Repair

There are two protocol options for introducing the mutation into yeast: the Quick Editing Protocol and the longer Transform Then Edit Protocol.

The Quick Editing Protocol induces the CRISPEY and Cas9 plasmids *during* the transformation step by adding inducer to the selection medium. The yeast cells that take up both plasmids immediately express the gRNA, repair template and Cas9. In lab strains, we have found that >90% of isolated colonies on transformation plates will have the desired edit. We do observe a decrease in transformation efficiency using this method, so it may not work for mutants or strains that are naturally difficult to transform.

The Transform Then Edit Protocol separates the transformation and editing steps. First, the CRISPEY and Cas9 plasmids are either sequentially or co-transformed into yeast. Selection is done in non-inducing conditions. Then, transformants containing both plasmids are grown in media containing inducer for 24-72 hr to introduce edits into the yeast genome. This protocol is significantly longer, but may be necessary for some wild strains.

Option 1 – Quick Editing Protocol

We highly recommend using the high efficiency transformation method developed by Carl De Boer. Detailed instructions below are a modification of the following published protocol:

- Carl De Boer 2017. High-efficiency S. cerevisiae lithium acetate transformation. protocols.io. https://dx.doi.org/10.17504/protocols.io.j4tcqwn

1. Select a pCas9-EcRT plasmid that has a different yeast selectable marker from the pCRISPEY plasmid containing your gRNA and repair template. Make sure both plasmids use the same induction system (galactose or β-estradiol).

2. Inoculate a 5 ml culture of the yeast strain you wish to edit. Incubate overnight at 30°C with shaking at 270 rpm.
    - For the GAL plasmids, use YP+2% galactose.
    - For the Z3 plasmids, use YPD (or any other medium that is appropriate for your strain).

3. Sub-culture into 100 ml medium to a starting OD600 of 0.05. Incubate at 30°C with shaking at 270 rpm until an OD600 of 0.3-0.4 is reached (~4-6 hr depending on strain growth rate and medium).
    - For the GAL plasmids, use YP+2% galactose.
    - For the Z3 plasmids, use YPD (or any other medium that is appropriate for your strain) containing 1 μM β-estradiol.

4. During incubation, prepare an ice bucket and place the following on ice:
    - 50% PEG 3350
    - 1M Lithium Acetate
    - 0.1M Lithium Acetate
    - 1M DTT
    - Sterile Water
    - Freshly denatured 2 mg/ml ssDNA (carrier DNA)
        - To denature, heat to 100°C for 5 min, immediately snap-cool on ice.

5. Collect cells by centrifugation at 3,000 rpm for 5 min. Pour off supernatant and place cells on ice. Keep cells on ice until heat shocked.

6. Resuspend cells with 1 ml cold sterile water and transfer to a 1.7 ml microfuge tube.

7. Centrifuge at 3,000 rpm for 2 min and pipette off supernatant.

8. Resuspend cells in 0.5 ml cold 0.1M Lithium Acetate. Centrifuge at 3,000 rpm for 2 min and pipette off supernatant.

9. Resuspend cells in as little cold sterile water as possible (generally ~50-100 μl). Pipette up and down to ensure all cell clumps are resuspended. The mixture will be very thick.

10. Prepare the transformation mixture on ice:

30 μl	denatured carrier DNA (2 mg/ml ssDNA)
140 μl	50% PEG 3350
22 μl	1M Lithium Acetate
2.12 μl	1M DTT
x μl	2-3 μg pCRISPEY plasmid containing gRNA+repair template
x μl	2-3 μg pCas9-EcRT plasmid
20 μl	yeast cells

11. Vortex gently to mix.
12. Incubate at 30°C for 30 min without shaking.

13. Heat shock in 42°C water bath for 20 min.

14. Centrifuge at 3,000 x g for 30 sec to pellet cells. Pipette off supernatant.

15. Resuspend cells in 100 μl of sterile room temperature water.

16. Plate entire volume onto the following plates:
    - For GAL plasmids using ANTIBIOTIC selection: plate on YP+2% galactose. Do not include antibiotics in plates at this step.
    - For GAL plasmids using AUXOTROPHIC selection: plate on SD+2% galactose plates lacking histidine and uracil.
    - For Z3 plasmids using ANTIBIOTIC selection: plate on YPD + 1 μM β-estradiol, or any desired medium containing 1 μM β-estradiol. Do not include antibiotics in the plates at this step.
    - For Z3 plasmids using AUXOTROPHIC selection: plate on SD + 1 μM β-estradiol plates lacking histidine and uracil. Any carbon source can be used for plates.

17. If AUXOTROPHIC selection was used, place plates in 30°C incubator for 2-4 days, until colonies appear.

18. If ANTIBIOTIC selection was used, place plates in 30°C incubator for 18-24 hr (overnight). The next day, replica plate to the following selective plates and incubate them for 2-4 days at 30°C, until colonies appear.
    - For GAL plasmids: plate on YP+2% galactose + antibiotics to select for BOTH plasmids.
    - For Z3 plamsids: plate on YPD + 1 μM β-estradiol + antibiotics to select for BOTH plasmids. Alternatively, any other medium containing 1 μM β-estradiol and both antibiotics can be used.

Option 2 – Transform Then Edit Protocol

1. Select a pCas9-EcRT plasmid that has a different yeast selectable marker from the pCRISPEY plasmid containing your gRNA and repair template. Make sure both plasmids use the same induction system (galactose or β-estradiol).

2. Transform the pCRISPEY plasmid containing your gRNA/repair template and the pCas9-EcRT plasmid into your yeast strain using your preferred method for yeast transformation.
    - For lab strains and strains with high transformation efficiencies, you can co-transform the pCRISPEY plasmid and pCas9-EcRT plasmid in a single transformation.
    - For wild strains that have lower transformation efficiencies, you may need to transform the pCRISPEY and pCas9-RT plasmids sequentially. After transforming the first plasmid, make sure to include selection for the first plasmid in all steps of the transformation procedure for the second plasmid to ensure the first plasmid will not be lost in the process of transforming the second plasmid.

- For antibiotic selection, plate transformations on YPD. Grow overnight at 30°C, then replica plate to YPD containing antibiotic selection for appropriate plasmid(s). Incubate selective plates 2-3 days until colonies appear.
- For auxotrophic selection, plate transformations directly on SC medium lacking Uracil and Histidine to select for both plasmids. Incubate 2-3 days until colonies appear.

3. Inoculate 8-10 isolated colonies from your transformation plate into 2 ml pre-induction medium. Only take a tiny amount of biomass from each colony to prevent your starting culture from being too dense.
    - For galactose-induced (GAL) plasmids, use SC + 2% Raffinose with selection for both plasmids. Raffinose is used as a carbon source because it will not induce or repress expression from the gal promoter.
    - For β-estradiol-induced (Z3) plasmids, the pre-induction medium is whatever medium you will be using for induction minus the β-estradiol. Make sure your medium contains selection for both plasmids. Any medium can be used with the β-estradiol system, including YPD or SC with any carbon source.
    - Any volume of medium may be used for the pre-induction and induction steps, but 2 ml should be sufficient in most cases.

4. Grow pre-induction culture for 24 hr at 30°C with shaking.

5. Transfer 60 ul of the pre-induction culture into 1.94 ml of induction medium.
    - For galactose-induced (GAL) plasmids, use SC + 2% Galactose containing selection for both plasmids.
    - For β-estradiol-induced (Z3) plasmids, use any medium containing selection for both plasmids and 1 uM β-estradiol.

6. Grow the first induction culture for 24 hr at 30°C with shaking. The gRNA and Cas9 will be expressed during this step to induce CRISPR/Cas9 cutting. The repair template will also be expressed and amplified by the Ec86 reverse transcriptase to increase the frequency of homologous recombination to repair the double stranded break introduced by Cas9.

7. Transfer 60 ul of the first induction culture into a new tube containing 1.94 ml of fresh induction medium.

8. Grow this second induction culture for another 24 hr at 30°C with shaking to continue inducing Cas9 cleavage for a total of 48 hrs.

9. Spot 5 ul of induction culture onto the edge of a petri plate, then streak for isolation on the rest of the plate. Alternatively, a serial dilution of the induction culture can be plated to obtain isolated colonies.
    - Any medium can be used for plating, however it is recommended to include selection for both plasmids in your plates to increase the proportion of edited colonies.

10. Grow plates 2 days at 30°C, until colonies are visible.

Note

It is highly recommended to include a control induction using the ADE2 gRNA plasmid when you are inducing a new plasmid for the first time. Select an appropriate ADE2 control plasmid that has the same induction system as your new plasmid and use the same pCas9 plasmid for both inductions. You can determine the editing efficiency of the ADE2 induction by dividing the number of red colonies by the number of total colonies on your induction plate. The ADE2 induction should yield >90% edited colonies by 48 hr.

Screening Isolates for Edits

They type of screen used to assess editing success will depend on the type of mutation being introduced into the strain.

- If the mutation being introduced has a known phenotype that is easy to test, that is often the most efficient screening method. In this case, it is recommended to screen ~20 isolates.
- If there is an easily differentiated genotypic difference between the mutation you are introducing and the wildtype allele, such as a difference in gene size, then PCR can be a useful screening method. In this case, we recommend screening 10-15 isolates.
- Point mutations can be more difficult to screen. If your point mutation disrupts or introduces a restriction site, PCR of that region followed by restriction digest may be a viable screening strategy. Alternatively, amplification of your gene of interest followed by sequencing can be used to verify the mutation. In this case, we recommend sequencing only 2-3 isolates and only sequencing more if the first few were unedited (to keep costs down). We do not recommend sequencing more than 10 isolates. In this case, it is more cost effective to re-try the induction.
- Make sure your screening method can differentiate between homozygous and heterozygous mutations if you are making your mutations in a diploid strain. Alternatively, diploid strains can be dissected to ensure the introduced mutation is homozygous.

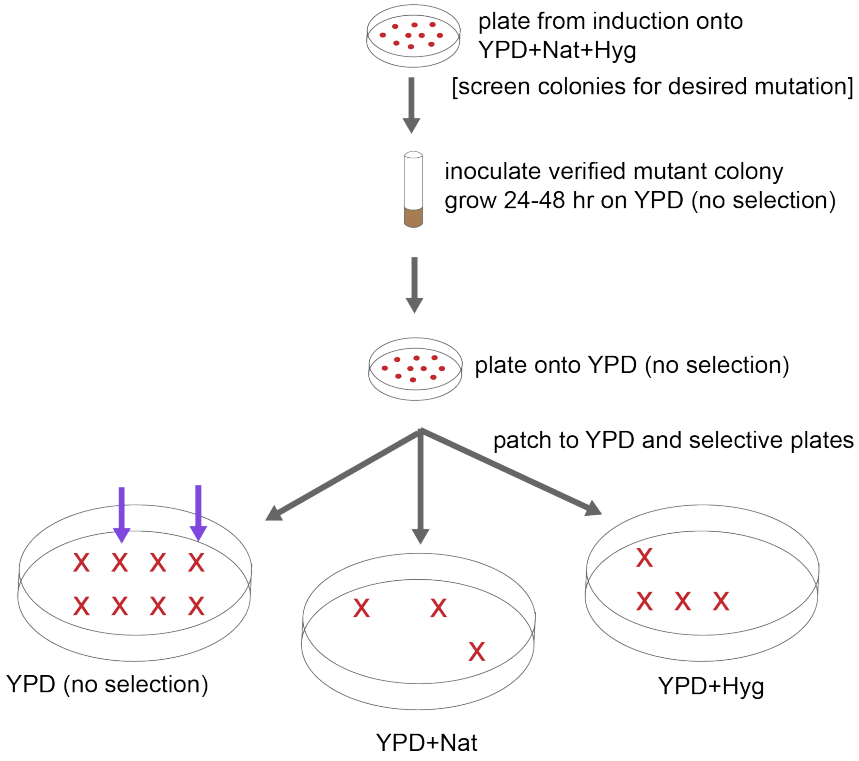
Curing Plasmids from Final Strains

1. Grow final strains non-selectively in YPD (no antibiotics) for 24-48 hr.
    - Any medium can be used to cure plasmids as long as it does *not* select for either plasmid.

2. Plate culture to get isolated colonies on any non-selective medium (generally YPD). This can be achieved by pipetting 5 ul of culture onto the edge of a plate and streaking for isolation across the rest of the plate, or by spread-plating a serial dilution of culture.

3. Patch ~20 colonies onto plates containing each antibiotic separately to check for plasmid loss. Also patch to a YPD plate. Isolates sensitive to both the pCRISPEY plasmid antibiotic and pCas9-EcRT plasmid antibiotic have lost both plasmids.
    - If you only find colonies that have lost one plasmid, you can repeat the YPD outgrowth and plating with isolates that have lost the one plasmid to cure the second plasmid sequentially.

Example
Introducing *ade2* deletion using pCRISPEY-GAL-Nat-ADE2 and pCas9-EcRT-GAL-Hyg plasmids



Colonies that have been cured of both plasmids are indicated by purple arrows

Troubleshooting

- **I am getting a weak or no band when amplifying my gRNA/repair template oligo.**
    - Try using CloneAmp HiFi PCR Premix (Takara cat# 639298).
    - Check that the concentrations of primers and template are correct.

- **I am getting low cloning efficiencies.**
    - If you are using another cloning kit, try the NEBuilder® HiFi DNA Assembly kit (cat# E5520S). We generally get >95% cloning efficiency with this kit.
    - Run your insert PCR product on a gel to make sure you are getting a single product of the correct size.
    - Run your digested vector on a gel to make sure your restriction enzyme is complete and only cutting the vector once. If you are getting a partial digest, consider adding more enzyme or purchasing a fresh tube of enzyme. If your enzyme is cutting the vector more than once, check your enzyme for star activity. You may need to reduce the incubation time or use a different version of your enzyme that is engineered to have lower star activity.
    - Try altering the amount of DNA in the reaction or altering the ratio of insert to vector. See the instruction manual for the cloning kit you are using for tips.
    - Try ordering shorter versions of the CRISPEY primers with smaller regions of homology for Gibson cloning. We have had success using 20 nt of homology.

- **Where can I find a protocol for transforming yeast?**
    - Gietz, R., Schiestl, R. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc.* (2007). Vol 2. Pages 31–34. https://doi.org/10.1038/nprot.2007.13
    - Carl De Boer. High-efficiency S. cerevisiae lithium acetate transformation. *Protocols.io* (2017). https://dx.doi.org/10.17504/protocols.io.j4tcqwn
    - Lorenzo Benatuil, Jennifer M. Perez, Jonathan Belk, Chung-Ming Hsieh. An improved yeast transformation method for the generation of very large human antibody libraries, *Protein Engineering, Design and Selection*. (2010). Vol 23. Pages 155–159. https://www.nature.com/articles/nprot.2007.13

- **I'm having trouble transforming plasmids into my yeast strain.**
    - We have noticed that some wild strains are more difficult to transform. Your transformation procedure may need to be optimized for your strain.
    - If using heat shock, some wild strains are temperature sensitive. Try lowering the heat shock time and/or temperature. You can also try skipping the heat shock and incubating your cells with the plasmid(s) in the transformation buffer overnight at room temp for the transformation.
    - For some wild strains, harvesting cells for transformation at a lower OD (at early log phase) can help.
    - Try scaling up both the number of cells and amount of DNA for your transformation.
    - Try different transformation protocols (see links above).

- **My yeast strain isn't growing (or grows poorly) during the induction steps.**
    - Try using the Quick Editing Protocol.
    - Some wild strains and mutants grow poorly in galactose as a sole carbon source. If this is the case, try the Z3 plasmids so you can use glucose as a carbon source, or your strain's preferred medium.
    - If you get little to no observable growth in the galactose induction, try spread plating 100 ul of the induction. We've gotten successfully edited isolates from plating inductions that appeared to have no growth at all in the induction cultures.
    - If using SC medium, check the age of your yeast nitrogen base. It can go bad and some wild strains seem to be more sensitive to this than lab strains.
    - Instead of sub-culturing 60 ul of cells into 1.94 ml of medium during the induction steps, measure the OD600 of your cultures and dilute them down to a starting OD600 of 0.2 at each induction step.
    - Try using SC+ 2% raffinose + 2% galactose as the induction medium.

- **None of the colonies I screen have my mutation.**
    - If you used the quick editing protocol, try the longer transform-then-edit protocol.
    - If you suspect the mutation you are trying to introduce has a general growth defect, try doing the quick editing protocol or plating the induction after only 24 hr. Your edited isolates may be dropping out of the population during the induction steps.
    - Try doing a third passage in the induction medium for a total of 72 hr induction. If the editing efficiency of your particular gRNA is low, this may help.
    - Make sure the mutation you are introducing does not have a growth defect in the induction medium. For example, if your mutation causes a growth defect in galactose, the induction medium will be selecting for wildtype cells. If this is the case, switch to the β-estradiol system so you can use a different induction medium that will allow growth of your mutant.

- Do a control induction side-by-side with your test induction using a control plasmid containing an ADE2 gRNA and repair template for introducing an frameshift in ADE2. Select the appropriate control plasmid that uses the same induction system and selection as the induction you are trying to optimize. You can determine the efficiency of the control induction by dividing the number of red colonies by the total number of colonies on your induction plate. If your ADE2 induction is working well, but your test induction is not, there may be something wrong with your pCRISPEY plasmid. If the ADE2 induction is not working, there may be something wrong with your induction medium or induction procedure. Note that the red colony color of the ADE2 deletion is easier to visualize on SC than YPD and increases in intensity over time.
- Check the sequence of your pCRISPEY plasmid to make sure there are no unwanted mutations, especially in the gRNA, repair template, retron recognition sequences, or promoter regions.

- **I'm having trouble curing my plasmids from my final strains.**
  - Try increasing the amount of time you grow the culture non-selectively before plating up to 72 hours. You can try sequential cultures (similar to the sub-culturing strategy for the induction steps).
  - Make sure the medium you are growing cultures in during the curing step does NOT select for either plasmid.
  - Consider replica printing final plate instead of patch plating so you can screen more colonies.
  - We have noted that some wild strains are more difficult to cure of plasmids than others and require longer incubation without selection.

Recipes

Synthetic Complete (SC)

1.7 g yeast nitrogen base without amino acids and without ammonium sulfate
1.0 g monosodium glutamate (MSG)
2.0 g amino acid mix (complete)

Bring volume to 1L and autoclave. Store at room temp for up to several weeks.

Note: it's important to use MSG as a nitrogen source because ammonium sulfate can interfere with some antibiotics

Amino Acid Mix (Complete)

0.54 g Adenine
0.24 g p-Aminobenzoic acid
11.4 g Leucine
2.28 g Alanine
2.28 g Arginine
2.28 g Asparagine
2.28 g Aspartic acid
2.28 g Cysteine
2.28 g Glutamic acid
2.28 g Glutamine
2.28 g Glycine
2.28 g Histidine
2.28 g myo-inositol
2.28 g Isoleucine
2.28 g Lysine
2.28 g Methionine
2.28 g Phenylalanine
2.28 g Proline
2.28 g Serine
2.28 g Threonine
2.28 g Tryptophan
2.28 g Tyrosine
2.28 g Uracil
2.28 g Valine

Place all dry ingredients in a coffee grinder.

Grind for 1-2 min while shaking grinder to thoroughly homogenize mix.

Store in a bottle of conical tubes at room temp, protected from light.

For auxotrophic selection, omit that amino acid from the mix. Medium is then called SD (synthetic dropout).

Antibiotics

Concentrations of antibiotics in final media:

Hygromycing B (use for HYG markers): 300 ug/ml
Nourseothricin (use for NAT markers): 100 ug/ml
G418 (use for KAN markers): 200 ug/ml

Concentrated stocks can be made at 1000x in water, filter sterilized and stored at -20°C for up to a year. It is highly recommended to aliquot stocks into 1 ml tubes before freezing to minimize freeze/thaw cycles.

β-Estradiol Stocks

Prepare stocks in glass vials. Store stocks at -20°C. Do not autoclave β-estradiol.

Concentrated stock: 50 mM β-estradiol in 100% Ethanol

- 0.01362 g β-estradiol in 1 ml 100% Ethanol

Working stock: 5 mM β-estradiol in 50% Ethanol

- 100 ul 50 mM β-estradiol (in 100% ethanol) stock
- 400 ul 100% Ethanol
- 500 ul sterile water

Add β-estradiol to sterile induction medium at a 1 uM final concentration just before use.